Inhibition of multidrug efflux as a strategy to prevent biofilm formation
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Co-ordinated regulation of multidrug efflux and biofilm formation in *Salmonella*.  
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* and Mark A Webber  
10 February 2016  
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Running head: Efflux and biofilm formation  
Keywords: curli, AcrAB-TolC, *ramA*
Abstract

Objectives. We have recently shown that inactivation of any of the multidrug efflux systems of *Salmonella* results in loss of ability to form a competent biofilm, the aim of this study was to determine the mechanism linking multidrug efflux and biofilm formation.

Methods. Mutants lacking components of the major AcrAB-TolC system were investigated for their ability to form a biofilm, aggregate and produce biofilm matrix components. The potential for export of a biofilm relevant substrate via efflux pumps was investigated as well as expression of genes that regulate multidrug efflux and production of biofilm matrix components.

Results. Mutants of *Salmonella enterica* serovar Typhimurium which lack TolC or AcrB but surprisingly not AcrA were compromised in their ability to form biofilms. This defect was not related to changes in cellular hydrophobicity, aggregative ability or export of any biofilm specific factor. The biofilm defect associated with inactivation of *acrB* or *tolC* resulted from transcriptional repression of curli biosynthesis genes and consequent inhibition of the production of curli by mutants lacking AcrB or TolC. This repression was associated with up-regulation of the global regulator, *ramA* and artificial over-expression of *ramA*, *marA* and *soxS* each decreased biosynthesis of curli, and inhibited biofilm formation. However, inactivation of these regulators did not rescue the ability of efflux mutants to form a biofilm.

Conclusions. This work shows biofilm formation and multidrug efflux are coordinately regulated, and that transcriptional repression of curli biosynthesis causes a lack of biofilm formation which occurs in response to lack of efflux activity or as a result of over-expression of global regulators *ramA*, *marA* and *soxS*. 
Introduction

Bacterial biofilms are a major clinical and industrial problem and eradication of biofilms presents a challenge for antimicrobial chemotherapy. Bacteria within a biofilm are encased within an extracellular matrix which commonly includes polysaccharides, proteins and other species specific components. Multidrug resistance efflux (MDR) pumps are transporters which can export a wide range of xenobiotics including antibiotics, dyes, biocides and other toxic molecules preventing lethal accumulation within the cell. The expression of efflux pumps is tightly regulated and efflux genes are usually subject to control by both local and global regulators. 

*Salmonella enterica* serovar Typhimurium (S. Typhimurium hereafter) has nine MDR systems from four separate protein families; the major system in *Salmonella* is the AcrAB-TolC RND system. The homologous global transcription regulators MarA, RamA and SoxS can all increase expression of *acrAB* in response to stress. We have recently described an inability to form a competent biofilm associated with inactivation of any of the MDR systems of S. Typhimurium including those which are normally cryptic in standard laboratory conditions. We found that production of curli, a major component of the *Salmonella* biofilm extracellular matrix was defective in all these strains, suggesting a common mechanism for the lack of biofilm formation in all mutants.

Here, using AcrAB-TolC as a paradigm we investigated the mechanism by which loss of efflux activity results in a lack of curli production. We ruled out export of a factor crucial for biofilm development via AcrAB-TolC and also showed that inactivation of components of AcrAB-TolC did not alter cellular hydrophobicity. However, inactivation of efflux components was found to significantly alter expression of biofilm related genes. We demonstrate that the biofilm defect of
mutants lacking AcrB or TolC is due to transcriptional repression of curli biosynthesis in the efflux mutants. Additionally, over-expression of the global regulators ramA, marA or soxS resulted in repression of curli biosynthesis and loss of biofilm formation. This work demonstrates a mechanism whereby loss of MDR efflux pumps impacts production of a biofilm due to co-ordinated regulation of efflux and biofilm formation.
Materials and methods

Strains and growth media

All strains used in this study and their origins are shown in Table 1. S. Typhimurium ATCC 14028S (L828) was used as a control strain throughout. Isogenic derivatives, L829 (tolC::cat) and L830 (acrB::aph) have been described previously,\(^6\) New mutants were created by transduction of mutant alleles into L828, and resistance marker cassettes were removed by plasmid pCP20 as previously described.\(^10\) To select for transductants, 100 µl from each transduction reaction was spread onto LB plates supplemented with 50 mg/L of kanamycin or 25 mg/L of chloramphenicol and incubated overnight at 37°C. Transfer of each mutant allele was verified by PCR and sequencing. Strains were stored at -20°C on Protect™ beads and routinely cultured on Luria-Bertani agar or broth unless stated otherwise. Over-expression and complementation plasmids containing \textit{marA}, \textit{soxS} or \textit{ramA} were constructed in pTRC and pWKS30 as previously described.\(^7\)

Biofilm formation assays

Various models were used to analyse biofilm formation in this study. For crystal violet biofilm assays, overnight cultures of strains were diluted in fresh Luria-Bertani broth without salt to an optical density of 0.1 at 600 nm. 96 well polystyrene microtitre trays (Sterilin) were inoculated with 200 µl of this suspension and incubated at 30°C for 48 hours with gentle agitation. After incubation liquid was removed from all wells and wells were washed with sterile distilled water to remove any unbound cells. Biofilms were stained by adding 200 µl of 1% crystal violet to appropriate wells for 15 minutes. Crystal violet was removed and each well washed with sterile distilled water to remove unbound dye. The stained biofilm was solubilised by adding 200 µl of 70% ethanol and optical density measured at 600 nm.
using a FLUOstar Optima (BMG labtech). All biofilm assays were repeated three times with two biological and four technical replicates per repeat.

To determine whether biofilm formation in L829 (\textit{tolC::cat}) and L830 (\textit{acrB::aph}) could be rescued by co-incubation with L828 (wild-type), strains were grown separated by a 0.45 µm membrane and biofilms formed as in the crystal violet assay but in 500 µl volumes in 24 well transwell plates. Assays were repeated with and without the presence of L828 (wild-type) in the upper ‘insert’ chamber with liquid contiguous between the upper and lower chambers. Biofilms were stained with crystal violet and quantified as above. Assays were repeated with addition of either a mid-logarithmic or stationary phase culture of L828 (wild-type) to assess whether growth phase had an impact upon production of any soluble biofilm promoting factor.

Biofilm formation under flow conditions were formed and visualised using a Bioflux microfluidic system (Fluxion) and phase contrast microscopy. Flow channels were inoculated with overnight cultures diluted in LB broth without salt to an optical density of 0.8 at 600 nm, plates were then incubated at 30ºC for three hours to allow the bacteria to adhere to the flow channels. Fresh LB broth without salt was then applied to the inlet wells of the plate and pumped through the flow cells at a force of 0.3 dynes at 30ºC for 48 hours. Phase contrast microscopy was used to visualise the biofilms formed at x10m, x20 and x40 magnification.

**Aggregation assays**

To examine whether loss of \textit{acrB} or \textit{tolC} led to alteration in cellular hydrophobicity or aggregative ability two different assays were used.

To measure the time taken for strains to settle, strains were incubated overnight in 10 ml LB (without salt) broths with shaking (150 rpm) before being placed statically on the bench. Samples (100 µl) were taken periodically from immediately below the
surface of the liquid and the optical density at 600 nm measured and recorded. Enteric aggregative E. coli O42 was used as a positive control. To determine whether there were any intrinsic differences in aggregative ability of each strain ammonium sulphate was used to induce aggregation of bacterial cells, a 4 M stock of (NH$_4$)$_2$SO$_4$ was made in 1 X PBS and adjusted to a pH 6.8. This stock was then serially diluted and mixed 1:1 (in 100 µl final volume) with bacterial suspensions (adjusted from an overnight culture to an OD 570 nm of 0.8) for each strain. These suspensions were immediately added to a microscope slide and rocked gently for 30 min before aggregation scored visually under a microscope as the presence of a precipitate. The lowest concentration of (NH$_4$)$_2$SO$_4$ required to induce aggregation was recorded for each strain.

**Artificial over-expression of ramA, soxS and marA**

pTrc-ramA carrying an IPTG inducible ramA was transformed into L828 (wild-type) to observe the phenotypic effects of over expressing ramA.$^{11}$ pTrc-soxS and pTrc-marA were constructed in a similar manner and introduced into L828 (wild-type), L829 (tolC::cat) and L830 (acrB::aph). Plasmid DNA was harvested from 10 ml cultures of strains containing plasmids after overnight incubation in LB broth at 37°C using the QIAlprep® Spin Cell Mini Kit (QIAGEN, U.K). The resulting plasmid DNA was analysed by agarose gel electrophoresis and quantified using Gene Tools software (Syngene, Cambridge, U.K). Plasmid DNA was transferred into recipient cells by electroporation. The impact of over-expression of each of the regulators on biofilm formation was investigated in the crystal violet assay; wells containing plasmids were supplemented with 100 mg/L of ampicillin and 1 mM IPTG to induce gene expression.

**Determination of gene expression**
The temporal and spatial expression of *ramA*, *marA* and *soxS* within biofilms was visualised using promoter-*gfp* fusion reporter constructs in pMW82. Strains carrying reporter plasmids were grown overnight in LB containing 100 mg/L of ampicillin then adjusted in PBS to an optical density of 0.1 at 600nm. Spots (5 µl) were inoculated onto LB – NaCl agar plates containing 100 mg/L of ampicillin and 40 mg/L of Congo red and incubated statically at 30 °C. Fluorescence of colonies was visualised after 24 and 48 h using a Nikon SMZ800 microscope (with Integilight C-HGFI fluorescence module attachment) and representative images captured. The expression of each regulator gene in response to addition of EIs was also inferred from measurements of fluorescence (Ex 487, Em 507) using a FLUOstar OPTIMA (BMG Labtech, U.K). Fluorescence was measured in a wild-type strain every 10 min over a 5 h period after the addition of a range of concentrations of the three EIs; PAβN, CCCP and chlorpromazine. Strains were grown in 100 µl of LB broth (inoculated with ~10^7 cfu/ml) at 30°C with shaking throughout the experiment. Induction of expression of each gene was calculated as the ration of average expression (based on 8 biological replicates) of induced samples compared to un-induced controls. The students ‘t’ test was used to determine significance of differences in *ramA* expression.

The expression of *marA*, *ramA*, *soxS*, *rob* and 16S rRNA were also determined by reverse-transcriptase PCR as previously described. The expression of *csgBAC* and *csgDEFG* were all determined using comparative RT-PCR, again as previously described. All primers used in this study are shown in Table 2.
**Staining of curli fimbriae**

Phenotypic differences in curli production were visualised by growing strains on agar containing Congo red (40 mg/L, Sigma-Aldrich Ltd., UK) and incubating them for 48h at 30°C as described previously. 9
Results

Mutants lacking a functional acrB or tolC do not form competent biofilms whereas a mutant lacking acrA is able to biofilm

A high throughput biofilm assay using crystal violet to stain cells adhered to a 96 well plate showed a significant decrease in the biofilm formation ability of L829 (tolC::cat) and L830 (acrB::aph) (figure 1). However, genetic inactivation of acrA (L1271 (acrA::aph)), the periplasmic adapter protein, had no negative effect on biofilm formation. The phase contrast microscopy images from biofilms formed in a flow cell under shear stress showed a similar pattern as the crystal violet assay with L828 (wild-type) forming a mature biofilm and L829 (tolC:cat) and L830 (acrB::aph) adhering as individual cells to the flow cell but unable to form a mature, three dimensional biofilm (figure 2).

Mutants lacking a functional acrB or tolC do not have an altered aggregative ability

To determine whether inactivation of AcrAB-TolC had altered the intrinsic aggregative nature of the strains lacking acrB or tolC a settle assay was used, this showed no significant difference in the aggregative ability of L829 (tolC::cat) or L830 (acrB::aph) (Figure S1A). Salt aggregation tests also showed no defect in the mutants’ ability to aggregate. In fact, L829 (tolC::cat) cells aggregated in a lower concentration of ammonium sulphate than L828 (wild-type) showing a slightly greater tendency for cells to aggregate than the wild-type (Figure S1B).

AcrAB-TolC does not export a factor required for biofilm formation

If a soluble biofilm promoting factor was exported by AcrAB-TolC, addition of culture supernatant conditioned by growth with L828 (wild-type) should be able to rescue the ability of the tolC and acrB mutant strains to form a biofilm. However, two co-
incubation assays with wild-type and mutant strains suggested that there is no ‘biofilm factor’ exported by AcrAB-TolC. Transwell assays showed the same poor ability to form a biofilm of the acrB and tolC mutants when incubated alone or co-incubated with L828 (wild-type) (Figure S2). In addition, no rescue of the biofilm defect was observed when co-incubated with logarithmic or stationary phase cultures of L828 (wild-type) (Figure S2). Similarly, biofilm mat assays co-inoculated with an equal ratio of wild-type and mutants showed that mutant cells did not comprise any of the biofilm mats formed, whereas the corresponding planktonic culture comprised an equal mixture of mutant and wild-type cells (data not shown).

**Expression of efflux and biofilm regulator genes differs between acrB and tolC mutants and an acrA mutant**

To explore the key observation that L1271 (acrA::aph) was not compromised in its ability to form a biofilm we compared the expression of genes known to regulate efflux gene and curli gene expression between this strain and the acrB and tolC mutants.\(^9,13\) This analysis revealed that ramA is significantly over-expressed in acrB and tolC mutants but not in the acrA mutant.\(^13\) Expression of ramA in these strains was also measured by RT-PCR and a ramA promoter-gfp fusion and results were consistent in showing up-regulation of ramA in the acrB (average ~2 fold) and tolC (average ~4 fold) mutants but not in the acrA mutant. The transcriptome of a ramA over-expressing strain was also investigated to identify alterations in expression of biofilm relevant genes; L786 (SL1344 pTrc::ramA) showed repression (two to five fold) of all the curli genes, including csgDEFG.\(^7,11\) This observation suggested that the biofilm defect in the acrB and tolC mutants was mediated by repression of curli biosynthesis and that this may be mediated by up-regulation of ramA.
The lack of curli production in mutants lacking acrB and tolC is due to transcriptional repression.

Congo red supplemented agar and Congo red staining of bacterial suspensions showed qualitatively and quantitatively that curli expression is repressed in the tolC and acrB mutants and produced at wild-type levels in the acrA mutant. This lack of curli production was found to result from transcriptional repression of various genes in the curli biosynthetic loci, as measured by cRT-PCR including the regulator csgD and all the structural and assembly genes also needed to produce curli. Expression of these genes was repressed in the tolC and acrB mutants but not in the acrA mutant (Figure 3).

Role of RamA, MarA and SoxS in repression of curli production and a loss of biofilm formation

To investigate whether RamA, MarA and SoxS are able to repress biofilm formation each was over-expressed in L828 (wild-type) and the consequences investigated. Artificial over-expression of ramA in L828 (wild-type) resulted in a complete loss of the ability of the strain to form a biofilm, over-expression of marA and soxS also resulted in a loss of biofilm formation although to a lesser extent than that seen with ramA (Figure 4). Production of curli on Congo red agar was repressed in each over-expression strain.

Spatial expression of ramA, soxS and marA within colonies of L828 (wild-type), L829 (tolC::cat) and L830 (acrB::aph) was visualised by fluorescence using regulator-gfp reporter plasmids. Figure 5 shows the pattern of expression seen for both ramA and marA was the inverse of where curli was being produced in each colony on Congo red agar. In wild-type colonies the highest ramA and marA expression was seen at the perimeter, where curli expression was at it's lowest. In the tolC and acrB mutant,
a higher level of ramA and marA expression is seen dispersed throughout the colony, again inversely correlating with phenotypic curli expression.

**Inactivation of the global regulators does not restore the ability of a tolC mutant to form a competent biofilm**

As all three regulators have the ability to repress biofilm formation when overexpressed and both marA and soxS are up-regulated upon inactivation of ramA, all three genes were inactivated to establish if rescue of biofilm formation would occur in tolC and acrB mutants. Inactivation of each of the regulators alone in the tolC and acrB mutants failed to rescue curli production and biofilm formation, however loss of each of the regulator genes was followed by consequent up-regulation of the others which may compensate for their inactivation (Figure 6). A series of multiple mutants lacking combinations of the three regulators also failed to rescue biofilm formation (Figure 7 shows the lack of rescue of the tolC mutant by loss of marA, soxS and ramA).
Discussion

Multidrug efflux pumps have a central role in the biology of bacteria with roles in drug resistance, cell division, pathogenicity and as recently described the formation of biofilms. Here, we investigated the mechanism which explains the inability of mutants lacking AcrB and TolC, constituents of the major AcrAB-TolC system of Enterobacteriaceae, to form a competent biofilm. Mutants of Salmonella lacking a functional tolC or acrB were unable to form biofilms under various conditions and this was not related to any defect in growth, cellular hydrophobicity/aggregative ability or export of a biofilm promoting substrate. Surprisingly a mutant lacking a functional AcrA (but still expressing AcrB), was not defective in its ability to form a biofilm. Loss of acrA has previously been shown to result in hyper-susceptibility to various drugs and a decreased ability to attach to and invade epithelial cells in tissue culture. The phenotype of an acrA mutant was, however, distinct from that of mutants lacking acrB. One major difference between acrA and acrB/tolC mutants is expression of the global regulator ramA, known to positively regulate expression of acrAB and tolC as well as other genes. Expression of ramA was up-regulated in both acrB and tolC mutants but not in an acrA mutant and analysis of microarray data showed that ramA up-regulation was associated with decreased expression of curli biosynthetic genes, this led us to hypothesise that curli repression was the reason that the efflux mutants did not form a biofilm and that ramA has a role in the co-ordinated regulation of efflux and biofilm formation. We confirmed the absence of curli production in mutants lacking tolC or acrB is due to strict repression of all the curli biosynthetic genes. We also confirmed that ramA over-expression repressed curli production and completely abolished biofilm formation. However, inactivation of ramA in the tolC and acrB mutants did not rescue their ability to form a biofilm. To
determine whether loss of ramA expression is compensated by other transcriptional activator genes, marA, soxS and rob expression was measured in mutant strains lacking ramRA, marRA or soxRS. Loss of ramRA resulted in increased expression of marA and soxS, both of which have some known functional overlap with RamA. Furthermore, over-expression of each of ramA, marA and soxS resulted in repression of curli production and biofilm formation. This suggests repression of biofilm formation and curli expression may be a core role for transcriptional activators that respond to stress and co-ordinate efflux up-regulation.

The regulation of curli expression is extremely complex with multiple pathways known to impact curli production. Amongst these pathways are two component systems which respond to membrane stress (cpxRA, rcsCB, envZ/ompR), rpoE and the lytic transglycosylases mltC and mltE. All these systems have membrane or periplasmic bound components demonstrating that curli synthesis is sensitive to changes in the membrane. Whether RamA/MarA/SoxS) act directly to repress curli synthesis or via one of the other currently known pathways which can influence curli repression is not known. Although there is a suggested RamA/MarA/SoxS consensus binding site, there is no good match for this sequence within the curli locus suggesting that the action of these regulators in mediating curli repression is indirect. Whilst we demonstrate here that over-expression of each of ramA, marA and soxS can repress biofilm we were unable to rescue the acrB and tolC mutants biofilm and curli production defect by inactivation of these genes individually or in combination. Therefore, while we have shown these regulators can repress biofilm formation in a manner phenotypically identical to that seen in response to loss of efflux it is unclear how important they are in mediating the biofilm defect seen in efflux mutants.
The demonstration that biofilm formation and efflux are co-ordinately but inversely regulated with loss of function of multidrug efflux resulting in repression of biofilm is interesting. Both de-repression of AcrAB-TolC and formation of a biofilm are in themselves protective against antibiotic action, the inverse regulation observed here seems counterintuitive. This relationship may act as a paradigm for other systems in other species where a link between efflux and biofilm formation exists. It is possible that conditions where efflux is up-regulated in response to stress represent a hazardous environment where formation of a biofilm and the subsequent attachment to a single site is a poor survival strategy explaining the evolution of a genetic switch between the two.
Acknowledgements

We thank Professor Dirk Bumann for the kind gift of pMW82 and are grateful to Professor Ian Henderson for kindly providing E. coli O42.

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Transparency declaration

None to declare.
References


Rosenberg, E. Y., Bertenthal, D., Nilles, M. L., Bertrand, K. P. & Nikaido, H. Bile salts and fatty acids induce the expression of *Escherichia coli* AcrAB


Figure legends

Figure 1. Crystal violet biofilm assay quantifies biofilm formation of L828 (wild-type), L829 (tolC::cat), L830 (acrB::aph) and L1271 (acrA::aph), showing genetic inactivation of tolC or acrB creates an inability to form a biofilm. Asterisks indicate significantly different average values to wild-type (p<0.05).

Figure 2. Phase contrast microscopy images of L828 (wild-type, panel A), L830 (acrB::aph, panel B) and L829 (tolC::cat, panel C) at 40 X magnification after 48 hours incubation under flow conditions.

Figure 3. Expression of curli genes. A schematic of the curli biosynthesis genes with average expression values determined by RT-PCR used to colour each gene showing repression in the tolC and acrB mutants. All expression values less than 50% of the wild-type were statistically significantly different (p<0.05).

Figure 4. Over-expression of ramA, marA or soxS represses biofilm formation. The bar chart shows biofilm formation in the crystal violet assay by L828 carrying pTrc-marA or pTrc-soxS or pTrc-ramA without or with induction with 1mM IPTG.

Figure 5. Expression of ramA and marA is up-regulated in efflux mutants and differentiated spatially within colonies shown by gfp reporter plasmids and correlates with a lack of curli production shown phenotypically on Congo red agar.

Figure 6. Inactivation of the ram, mar or sox loci results in compensatory up-regulation of redundant regulators. The graph shows average expression data from RT-PCR in each mutant and shows that when ramRA is inactivated in L828 (wild-type) there is a large increase in expression of marA and soxS to compensate.

Figure 7. Crystal violet biofilm assay shows loss of ramA, marA, soxS and combinations thereof does not rescue tolC mutant’s biofilm defect. Asterisks indicate significantly different average values to wild-type (p<0.05).
## Table 1. List of strains used in this study.

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<th>Genotype</th>
<th>Description</th>
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### Plasmid

- **pTrc-marA**: *marA* over-expression plasmid
  - This study
- **pTrc-ramA**: *ramA* over-expression plasmid
  - 7
- **pTrc-soxS**: *soxS* over-expression plasmid
  - This study
- **pWKS30-marA-gfp**: *marA* gfp reporter plasmid
  - This study
- **pWKS30-ramA-gfp**: *ramA* gfp reporter plasmid
  - This study
- **pWKS30-soxS-gfp**: *soxS* gfp reporter plasmid
  - This study
Table 2. Primers used in this study.

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Figure 1

![Bar chart showing biofilm formation (OD 600) for different strains.](image-url)

- L828 (wild-type)
- L829 (troC::cat)
- L830 (acrB::aph)
- L1271 (acrA::aph)

The chart indicates significant differences in biofilm formation among the strains.
Figure 2

A  B  C
Figure 3

L828 (wild-type)

L829 (tolC:aph)

L830 (acrB:aph)

L1271 (acrA:aph)

Key (Expression relative to wild-type):

- >75% wild-type expression
- 50-75% wild-type expression
- 25-50% wild-type expression
- 0-25% wild-type expression
Figure 4

![Graph showing percentage biofilm formation for different genes.]
Figure 5

Wild-type  tolC::cat  acrB::aph

Congo red morphology

ramA-gfp

marA-gfp
**Figure 7**

![Graph showing fold change in biofilm formation.](image-url)
SUPPLEMENTARY FIGURES

Figure S1.

Panel A. Settle assay of L828 (wild-type), L829 (tolC::cat) and L830 (acrB::aph). *E. coli* O42 was used as a positive, aggregative control. Values indicate the percentage of an initial absorbance from readings taken immediately below the surface of the liquid of a broth which was incubated statically over a 24h period.

Panel B. Salt aggregation test images of L828 (wild-type), L829 (tolC::cat) and L830 (acrB::aph) in 1M and 2M ammonium sulphate. Aggregation was recorded as formation of a visible precipitate and the lowest concentration of ammonium sulphate to prompt precipitation recorded for each strain.

Figure S2. Transwell assays show no rescue of biofilm formation by mutants when co-incubated with wild-type. (A) shows strains incubated with and without the presence of L828 inoculated at the same density as the mutants, (B) shows the same experiment but with co-incubation with an overnight, undiluted culture of L828.
Figure S2

A

Biofilm formation (relative to L828)

B

Biofilm formation (relative to L828)

L828  L830 (acrB::cat)  L829 (tolC::cat)  L828  L830 (acrB::cat)

+ wild-type supernatant

Strain